HUMAN GENETIC AND MICROBIAL FACTORS CONTRIBUTIONS TO THE DEVELOPMENT OF HIV-ASSOCIATED NEUROCOGNITIVE DISORDER

by

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BS in Biology, Barry University, 2011

Submitted to the Graduate Faculty of

the Department of Infectious Diseases and Microbiology

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2016

UNIVERSITY OF PITTSBURGH

Graduate School of Public Health

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ABSTRACT

Years of research and the development of effective therapeutic treatments, have dramatically improved the life expectancy rates for HIV-infected individuals. However, there is a subpopulation of aging, infected individuals who have experienced an adverse impact on their long-term health and quality of life, the mechanism of which has become an increasing concern of public health importance. The counteractive outcomes of aging within the infected population leaves many susceptible to developing age related morbidities in the form of cognitive impairment, brain atrophy, and other neurocognitive disorders at an earlier age then those within the non-infected population. These symptoms manifest in the form of HIV-associated Neurocognitive Disorder or HAND in infected individuals. Fully understanding the process in which HAND can occur has been a striving goal within the Public Health community. Our goal is to determine if there are specific genetic and/or microbial factors within individuals that may be contributing to their development of cognitive decline. All these efforts could provide comprehensive insight at an endophenotypic level into the pathological mechanism of HAND, and a better understanding of how diversity in the gut microbiome can affect health and aging. Subsequently, this information could lead to the identification of genetic biomarkers, development of treatments, and therapeutic options for regulating chronic HIV infection and neuropathology. We hypothesize that inherited SNPs in genes of the folate metabolism pathway affect the availability of methyl groups within the cell, and consequently influence DNA

methylation, leading to the development of HAND in seropositive individuals, and neurocognitive decline in seronegative individuals. We also hypothesize that there is an altered composition of the microbiome within the gut of infected individuals, the presence of which directs the level of HIV pathogenesis and HAND development. In comparing HIV+ and Cognitive Decline groups against control groups, we do not have sufficient evidence to conclude that there is an increased risk of adverse outcome in association with any of the folate genes that we observed. Isolation of bacterial genome produced expected PCR product, and data interpretation following 16S rRNA sequencing will soon yield definitive microbial composition analysis.

Keywords: HAND, DNA Methylation, HIV-Infection, Chronic HIV Infection, Long-Term ART Treatment, Accelerated Aging, Microbial Composition, Microbiota, Microbial Factors and Cognitive Decline, Genetic Factors and Cognitive Decline, Endophenotype

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1.0 INTRODUCTION

Since its initial discovery in the early 80's, HIV has grown into a global epidemic that has affected around 78 million people and lead to over 39 million deaths. Currently a little over 35 million people are living with the disease (WHO, 2016). The high incidence of infection has led to increased efforts in HIV research; resulting in a better understanding of HIV pathogenesis and leading to the development of efficient drug treatments. All these efforts contributed to decreasing the incidence of new infections globally and transforming HIV infection into a chronic and manageable disease. Since its introduction in the 90's, combined antiretroviral therapy (cART) has served as an effective therapeutic treatment for HIV, decreasing morbidity, mortality, and dramatically improving life expectancy. Current antiretroviral therapies work by suppressing viral replication and inhibiting disease progression. According to UNAIDS, antiretroviral therapy has contributed to the addition of 11.7 million years to infected individuals globally (UNAID, 2016). During the pre-cART era, individuals with advanced HIV prognosis along with severe immunosuppression developed a progressive subcortical dementia termed AIDS dementia complex or AIDS encephalopathy. But successful antiretroviral therapy has led to a dramatic decrease in the development of HIV-associated dementia within the infected population and reduced the risk of opportunistic infections in the central nervous system (CNS). Even with all this advancement in research and success in treatment; there is still a subpopulation of aging, infected individuals who have experienced a milder form of neurocognitive decline

with an adverse impact on their long-term health and quality of life even in the presence of active viral suppression**,** implying that HIV has neurocognitive effects on the brain and aging (Simoes & Justino, 2015). The counteractive outcomes of aging within the infected population leaves many susceptible to developing age related morbidities in the form of cognitive impairment, brain atrophy, and other neurocognitive disorders at an earlier age (before 60) then those within the non-infected population (Rickaboagh, 2015). The attributing cause of this phenomenon has yet to be definitively linked to either the long-term effects of antiviral treatment or chronic HIV infection.

1.1 HIV-ASSOCIATED NEUROCOGNITVE DISORDER (HAND)

1.1.1 Classification and Clinical Manifestation

HIV-associated Neurocognitive Disorder or HAND is a major neurocognitive disorder that plagues long-term infected individuals {Clifford, 2013}, serving as the leading neurological complication caused by HIV-1 infection (Simoes & Justino, 2015). A variety of clinical symptoms manifest during the shifting stages of HIV infection: the neurological complications associated with HAND include cognitive, motor, and behavioral symptoms. The gravity of the the pathology, severity of symptoms, and impact on quality of life are used as measures to determine the type of HAND is affecting an individual. Using the classification system, Frascati criteria, HAND is broken down into the following subsets:

1. Asymptomatic Neurocognitive Impairment (ANI): acquired cognitive impairment without any functional impairment

2. Mild Neurocognitive Disorder (MND): functional impairment with mild interference; enough to interfere with their working ability, cause reduced reasoning and ability to understand
3. HIV Associated Dementia (HAD): functional impairment that interferes with daily functioning

ANI and MND are the more prevalent forms of HAND and also the most difficult to define. Both conditions are defined by their level of functional impairment, however, due to limitations in testing and confounding factors, analysis is subjective and an exact diagnosis between the two disorders may be imprecise (Simoes & Justino, 2015).

1.1.2 Pathogenesis

HAND manifests when HIV spreads from the initial site of infection to the nervous system. The exact mechanism in which neuro-pathogenesis evolves into HAND has yet to be thoroughly identified; however, one commonly accepted theory is that HIV "traffics" into the CNS by hitchhiking on activated monocytes and lymphocytes, during the the early stages of exposure and infection (French, 2009). The systemic spread of HIV to the CNS leads to tissue damage and pathological changes in the basal ganglia, the deep white matter, and the hippocampus. Though systemic infection of HIV in the CNS and cerebral spinal fluid (CSF) occurs during early infection, not all occurrences of HIV infection in the brain leads to the development of HAND. Chronic CNS infection, persistent viral replication, inflammation, and neuronal injury and impairment have all been linked as risk factors in the pathogenesis of HAND (Simoes & Justino, 2015).

1.2 GENETIC EFFECTS ON PATHOGENESIS

In a previous experiment monitoring DNA methylation changes in HIV positive men with cognitive decline; whole genome methylation profiles were completed on a group of men from a long term longitudinal study (Pitts men Study-PMS). The measurements used in this study, examined the extent of methylation changes in individuals between two time points that were 10 years apart. The authors found a distinctive perturbation of methylation in individuals with cognitive decline, both in seropositive men with HAND and in seronegative men with comparable cognitive decline. They also found that the seropositive samples with HAND were on average fifteen years younger than the seronegative men with comparable cognitive decline. The data suggest that there was a noticeable methylation response associated with the presence or absence of cognitive decline, suggesting that DNA methylation could serve as a biomarker for cognitive changes in both seropositive and seronegative individuals. Methylation changes were also monitored amongst seropositive and seronegative individuals who did not show cognitive decline, however there was no significant difference in methylation between them (Martinson, 2015).

Recently, there has been other evidence linking DNA methylation to the development of HAND and studies have associated accelerated aging during HIV infection to site-specific changes in methylation patterns {Rickabaugh, 2015}. It is suggested that genetic variants may influence the host's susceptibility and progression rate of neuropathogenesis {Kallianpur, 2014}. In a more recent paper by Gross et al; they found that chronically infected pateints exhibited increased methylation changes at age associated methylation sites and both recently and chronically infected pts, had an average age-acceleration of 4.9 years, suggesting that it is HIV infection and not the length of infection is assoicated with accelerated ageing. All of this suggest

that CpG DNA methylation in the blood correlates with accelerated ageing and can serve as a signature a epigenetic tool (Gross, et al., 2016).

DNA methylation is an essential process that drives cell differentiation, healthy embryonic development, and gene regulation; reactions result in the addition of a methyl group to the cysteine nucleotide, altering gene expression and function (Bailey, 1999). Several genes are involved in the maintenance of methylation in cells, but vitamins like folic acid, are imperative for the synthesis of DNA. More specifically, Folate metabolism plays a significant role in the synthesis of S-adenosylmethionine (SAM), which is a methyl group donor in various methylation reactions, including the methylation of DNA {Nazki, 2014}. Additional polymorphisms have been characterized in other genes involved in folate metabolism, such as cystathionine beta-synthase (CBS), methionine synthase (MS), and methionine synthase reductase (MTRR), but their functional roles have not yet fully been characterized (Rajagopalan, et al., 2012).

Several pathways rely on folate metabolism to maintain its processes as depicted in **Figure 1 Folate Metabolism Pathway**, (Human Pathology , 2003) however deficiencies in folate modulation has attributed to the development of numerous diseases (depression/ dementia) and cellular dysfunction (DNA damage, compromising neuronal integrity). As stated previously; the presence of one enzyme in particular, S-adenosylmethionine (SAM), relies on the efficiency of folate metabolism. Folate deficiencies hinders S-adenosylmethionine's ability to donate methyl groups to important biosynthetic reactions, impacting neuronal homeostasis, and increasing the risk for neuropathologies. Specifically, decreased levels of S-adenosylmethionine in the brain and in the cerebral spinal fluid have been discovered to be associated with Alzheimer's disease, the most common form of dementia (Linnebank, et al., 2010). Deficiencies

in folate metabolism also leads to increased levels of circulating homocysteine; the presence of which has been associated with advanced aging, vascular risks, and cerebrovascular and neurodegenerative changes (Selley, 2007).

Folate metabolism is influenced by several factors including dietary intake of folic acid and polymorphisms of associated genes. Aberrant folate metabolism leads to abnormal distribution of methyl groups, reduced DNA stability, and consequent development of various diseases {Bailey, 1999}. Polymorphism within the genes involved in folate metabolism has been implicated in the development of a variety of metabolic and developmental disorders. The most well-known variant is the C677T mutation (rs1801133) in the methylenetetrahydrofolate reductase gene *MTHFR*, which causes an alanine-to-valine substitution in the MTHFR protein. The valine-containing variant is thermolabile and has reduced enzyme activity. This mutation has been implicated in the development of neural tube closure defects in pregnancy, and of cardiovascular disease later in life. Recently, it has also been found to be associated with mild cognitive impairment in two cohort studies (Rajagopalan, et al., 2012).

With what is understood, DNA methylation is used as a screening method for cancers, neurological disorders and age determination; however, it is not a defined method to monitor HAND development.

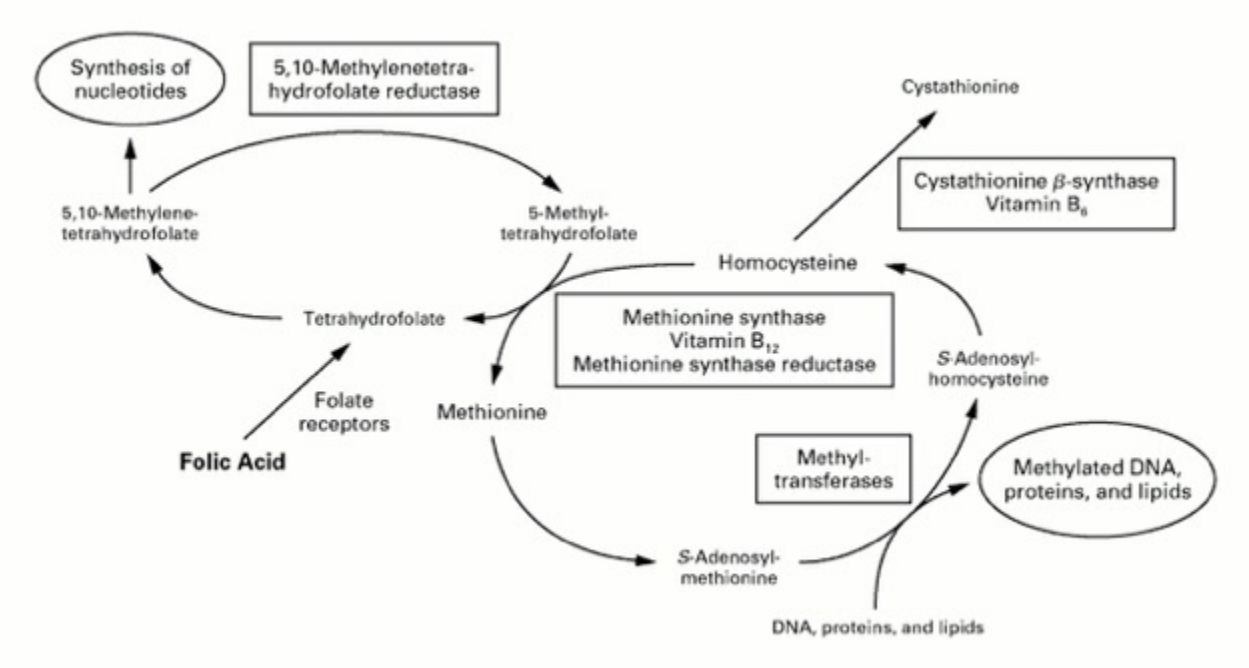

Figure 1 Folate Metabolism Pathway, (Human Pathology , 2003)

The preferred method of neurocognitive screening is MRI brain resonance imaging. MRIs are sensitive to identifying white matter and meningeal diseases (Cryan & Dinan, 2012); however, this technique isn't useful in identifying early stages of inter-cranial opportunistic diseases in pre-symptomatic HIV patients. Early diagnosis leads to better prognosis, so this process is not an option to be used to identify early stages of HIV-associated neurological disorders, therefore another method needs to be identified.

Studying genetic variation, such as single nucleotide polymorphisms (SNPs), in folate genes could begin a molecular investigation that could provide comprehensive insight at a genomic level to understand the causal mechanisms of HAND, and to aid in identifying specific markers and precursors to disease.

Based on these findings we hypothesize that Mendelian inherited SNPs in genes of the folate metabolism pathway affect the availability of methyl groups within the cell, and consequently influence DNA methylation that leads to the development of HAND in seropositive

individuals, and of neurocognitive decline in seronegative individuals. We further hypothesize that these SNPs may serve as biomarkers of neurocognitive decline risk in these individuals.

1.2.1 Specific Aim #1

We investigated this hypothesis by identifying several candidate SNPs in genes implicated in folate metabolism, DNA methylation, and the development of cognitive decline, and determining their allele frequency distributions in HIV seropositive and seronegative individuals from the Pitt Men's Study who exhibit symptoms of cognitive decline, matched with seropositive and seronegative controls who do not show these symptoms.

1.3 HIV INFECTION AND NEUROPATHOLOGY

HIV infection induces a systemic and progressive immunodeficiency that damages the mucosa, lymphoid tissues, and the brain. The damage results in neuropathological changes, immune dysfunction, and inflammation. During acute infection, HIV can cross the blood brain barrier through infected cells and infect the CNS. The virus continues to spread to other cells (microglia, astrocytes, etc.) (Nightingale, et al., 2014). In response to HIV in the CNS, monocytes become activated, inducing the release of proinflammatory cytokines and neurotoxins. Viral replication in the brain and responses to the infection cause neuronal damage that leads to impaired cognitive and motor ability, speech and behavioral changes, and memory dysfunction in infected individuals (Kallianpur, 2014). Even under the controlled regimen of antiretroviral therapy and inhibited viral replication; infected adults can still exhibit persistent

systemic inflammation due to latent reservoirs and a higher frequency of activated adaptive and innate immune cells {French, 2009}. Chronic inflammation is considered to affect the accelerated aging and morbidity seen within the infected population.

1.3.1 Gut Microbiome and HIV Pathogenesis

The gastrointestinal tract and its mucosal environment is heavily saturated with HIV target cells. During the systemic spread of HIV infection, the virus migrates through the Gut-Associated Lymphoid Tissue (GALT) and infects $CCR5^{+}CD4$ T cells. The subsequent loss of CD4 T cells in the gut reduces mucosal integrity and indirectly causes epithelial injury. The damage to the gut mucosa includes dysbiosis, microbial translocation, and loss of critical immune cells within the mucosa {Deeks, 2013}.

1.3.1.1 Dysbiosis

Under normal conditions the gut consists of commensal bacteria that aid in metabolizing nutrients in the food we eat. The normal enteric microbiota also supports healthy symbiosis and immune surveillance between microorganisms and the host (Sommer, 2014). Other properties of a healthy gut include protective mucosa, antimicrobial peptides, resident immune cells, and secreted antibodies. During infection, the increased release of proinflammatory and microbial products activates the IDO pathway, inhibiting the differentiation of TH17 cells and causing a shifting loss of IL-17 and IL-22 T cells {Deeks, 2013}. These immune cells are important for maintaining epithelial integrity in the gut; the loss of these cells impairs the guts antimicrobial properties and induces tissue damage. The depletion of these immune cells also results to an enhanced levels of bacterial species and increased microbial translocation.

1.3.1.2 Microbial Translocation

Due to the enhanced loss of mucosal integrity and the breakdown of tight epithelial junctions, all other protective barriers within the gut mucosa become compromised creating an ideal environment for microbial translocation to occur. Proinflammatory products made up of bacteria and fungi and metabolites are systemically released into circulation and migrate to local tissue (Stilling, 2014). The microbial products enter the portal vein and then travel to the liver. The presence of the microbial pathogens are then sensed by antiviral immune cells in the liver; those same cells then activate the proinflammatory and profibrotic pathways resulting in chronic inflammation and liver dysfunction {Duffield, 2005}. Microbial translocation decreases immune surveillance, impairs lymphoid tissue function, and induces peripheral circulation of microbial products while also inhibiting clearance of these products.

So what are the underlying causes of age related morbidities and neurocognitive disorders in HIV infected individuals?

1.4 GUT MICROBIAL COMPOSITION AND HIV NEUROPATHOGENESIS

The link between microbial composition and altered function in behavior and cognition has led to the development of the concept of microbiota-gut-brain axis {Stilling, 2014}. The gut microbiota influences the host's behavior and CNS activity by decreasing synaptic connection and promoting pain and anxiety perception {Sommer, 2013}. Increased presence of inflammatory cells and intestinal dysbiosis interferes with microbiota-gut-brain signaling; effecting behavior, cognition, and emotion {Moloney, 2014}. This process is depicted in **Figure 2 Microbiota-Gut-Brain Axis** (Cryan & Dinan, 2012). Understanding how diversity in the gut

microbiome can affect health and aging have led to the development of treatments and provided options for regulating chronic HIV infection and neuropathology Hypothesis: We hypothesize that there is an altered composition of the microbiome within the gut/stool of infected individuals, the presence of which directs/guides the level of HIV pathogenesis and HAND development.

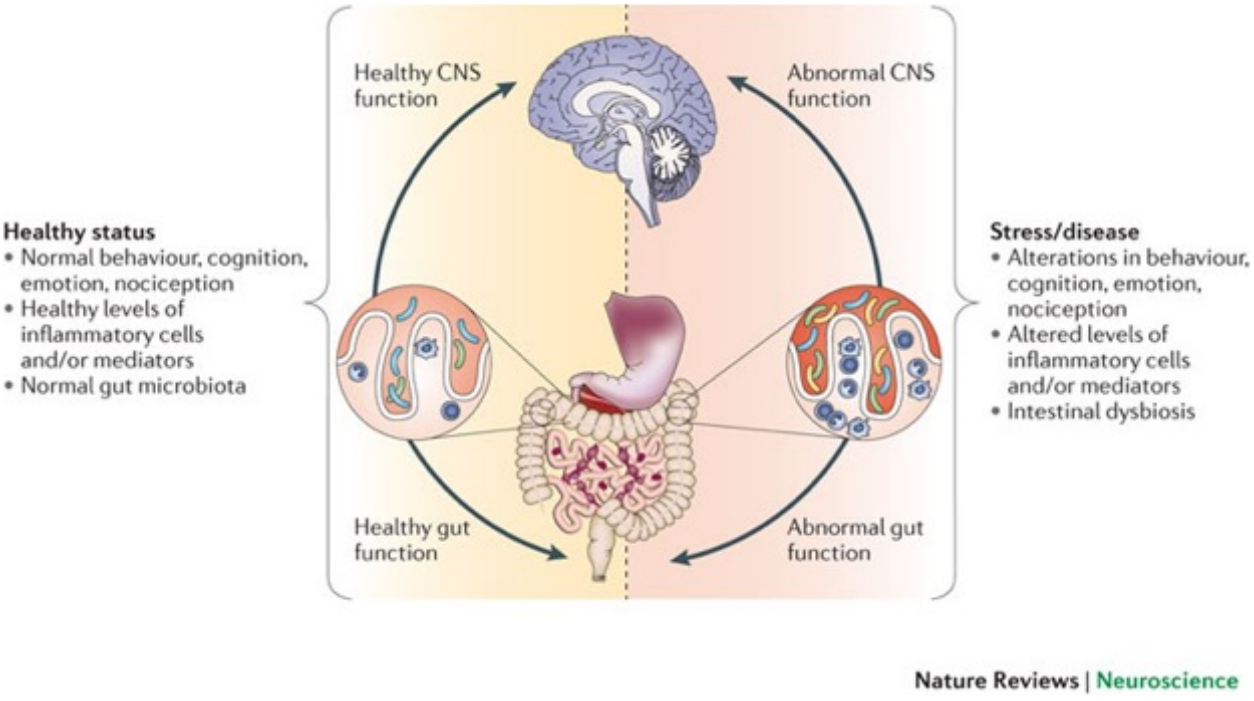

Figure 2 Microbiota-Gut-Brain Axis (Cryan & Dinan, 2012)

1.4.1 Specific Aim #2

We investigated this hypothesis by isolating microbial DNA from stool samples collected in the pre-cART era from men within the Pitt Men's Study who later went on to become infected with HIV and develop HAND, and also from men who remained seronegative. The composition of these DNA samples will then be determined by 16S rRNA subunit sequencing.

2.0 RESEARCH DESIGN

2.1.1 SNPs in genes involved in Folate Metabolism and DNA Methylation

2.1.1.1 Subject Sampling

DNA Samples: PBMC pellets were prepared from blood collected from male subjects enrolled in the Pitt Men's Study, the Pittsburgh branch of the Multicenter AIDS Cohort Study (MACS). The MACS is a multicenter (Baltimore, MD; Chicago, IL; Pittsburgh, PA; and Los Angeles, CA) ongoing prospective study, founded in 1984, of the natural and treated histories of HIV-1 infection in men who have sex with men. Participants attend clinics bi-annually for a physical exam and sample collection, and complete extensive questionnaires about their medical history, behavior changes, and overall quality of life. All samples were obtained from volunteer participants who had read and agreed to the consent policy implemented by the MACS for the protection of human subjects, and approved by the Institutional Review Board (IRB) at each MACS site. Study participants were separated into the following groups (n=86):

- HIV^-/Cognitive $Decline^-$ (n = 16)
- HIV^-/Cognitive $Decline^+$ (n = 12)
- HIV^+/Cognitive $Decline^-$ (n = 26)
- HIV^+/Cognitive $Decline^+$ (n = 26)

2.1.1.2 Extraction and Purification of Genomic DNA

Genomic DNA was extracted from PBMC pellets using the QIAGEN® QIAamp DNA Blood Mini Kit, and following the manufacturer's protocol. The process uses silica-membrane technology, to selectively purify DNA and filter samples through a fast spin column. This process was completed previous to my arrival to the lab. DNA was available at a concentration of 5 ug/mL for genetic analysis.

2.1.1.3 SNP Selection

Based on a review of the literature, nine single nucleotide polymorphisms (SNPs) in genes involved in folate uptake and metabolism were selected for analysis;

- 5-methyltetrahydrofolate homocysteine methyltransferase [**MTR]:** rs1805087
- Methionine synthase Reductase [**MTRR**]: rs1801394
- Methylenetetrahydrofolate Reductase [**MTHFR]**: rs1801133, rs1801131
- Cystathionine beta-Synthase [**CBS]:** rs234706
- Methylenetetrahydrofolate dehydrogenase 1 [**MTHFD1]:** rs2236225
- Nuclear Factor, Erythroid 2-like 2 microRNA 3128 [**MIR3128/NFEL2]:** rs1806649
- Glutathione S-transferase Omega 2/ MicroRNA 4482 [**GST02/MIR4482-1]:** rs4925
- Transcobalamin II [**TCN2]:** rs1801198

TaqMan® assays were obtained for each of these SNPs from Applied Biosystems (Foster City, CA).

2.1.1.4 TaqMan® SNP Genotyping Assays

The assays were prepared using TaqMan Genotyping Master Mix (Applied Biosystems) and the SNP assays previously listed (**Table 14. TaqMan SNP Genotyping Assay List**) (Applied Biosystems). The reaction mix was prepared for each reaction using the following: 5.00 ul of 2X TaqMan® Master mix, 0.15 ul of 20X working stock of Genotype Assay, and 4.85 ul of Nuclease-Free water. 10 ul of master mix and 1 ul of genomic DNA at a concentration of 5ug/mL, was placed into each respective well. Plate was then vortexed and spun before being placed into a Life Technologies StepOne Plus Real-Time PCR system.

Table 1. Real-Time PCR/QPCR

	Per Sample	**Total**
Genotype Master Mix (TaqMan)	5.0ul	500ul
MTHFR Primer Probe	.15ul	15ul
DiH_20	4.85	485ul
***DNA* sample (5ug/ml)**	1ul	

2.1.1.5 PCR Plate Read and Analysis

Raw fluorescent data was collected at the end of each cycle of the PCR process. Allele specific TaqMan probes with linked reporter dyes FAM™/SYBR® Green, VIC®/JOE™ detected alleles specific to the polymorphism of interest. ROX™ dye was included as a passive reference.

The StepOne™ Software v2.1 was used to determine the location and intensity of the fluorescent signals in each read, the dye associated with each fluorescent signal, and the

significance of the signal. The raw data was then graphed into a scatter plot using Microsoft Excel.

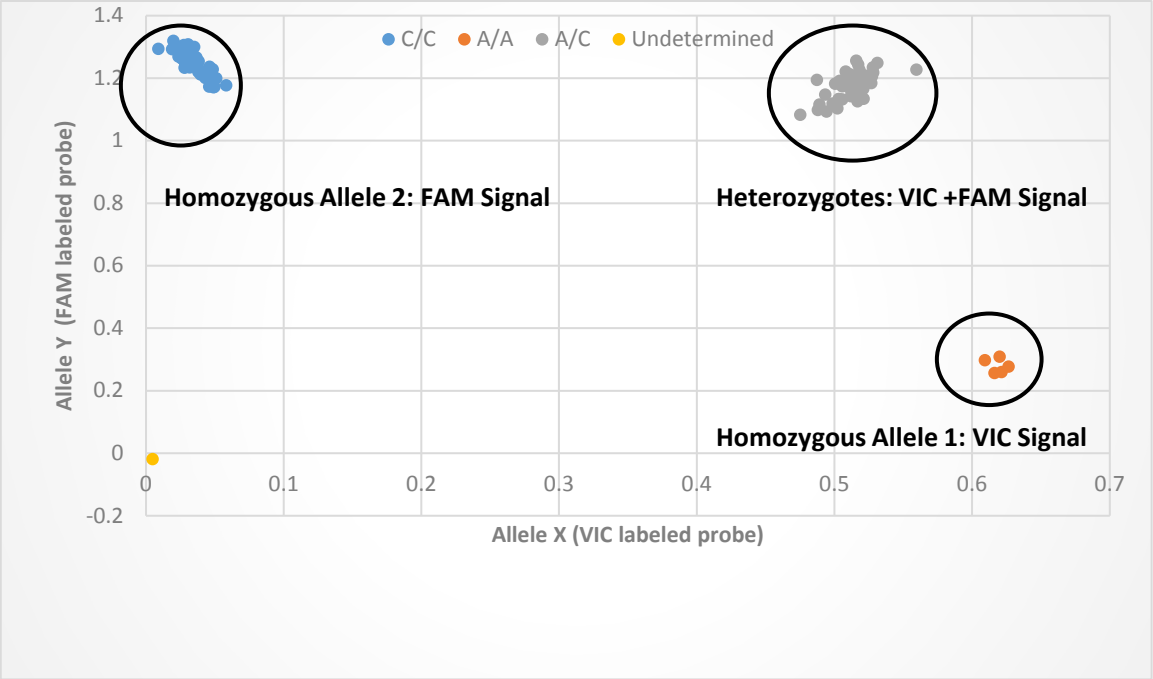

Figure 3 Allelic Discrimination Plot (Victor, 2015)

2.1.1.6 PCR Amplification of Genomic DNA

DNA was selectively amplified via touchdown PCR. All PCR preparations were completed on ice, gently mixed, quickly spun, and then placed in Mastercycler® Gradient (Eppendorf) to complete the following program: Initial denaturation at 95°C for 2 minutes, followed by cycles consisting of denaturation at 95°C for 30 seconds, annealing at 61°C for 30 seconds, and elongation at 72°C for 1 minute. These cycles were repeated another 13 times with an decrementing annealing temperature of 0.05°C per cycle. The samples were then denatured at

95°C for 30 seconds, annealed at 57°C for 30 seconds, and elongated at 72°C for 1 minute, for a further 22 cycles.

Table 2. PCR Master mix

MATERIALS	PER SAMPLE
5 ug/ml of DNA sample (previously diluted)	2 ul
10x Buffer	2.5 ul
25 mM $MgCl_2$	1.5 ul
25 mM dNTPs	0.2 ul
10 mM Primers	0.4 ul
Taq Polymerase	0.2 ul
DiH_2O	18.2 ul
TOTAL	25 ul

DNA yields were confirmed using gel electrophoresis.

2.1.1.7 Sanger Sequencing

Due to its poor separation of genotyping clusters on the TaqMan assay, we performed a Sanger Sequencing protocol on one of the SNPs in our profile (rs1801133-MTHFR C677T). The protocol was executed in the following order, on the PCR product prepared as described in the previous section:

1. Alkaline Phosphatase/Exonuclease I Digestion: This process removed any excess primers or dNTPs within our DNA template. A master mix of the following contents were prepared on ice:

Table 3. Exo-SAP Master Mix

Materials	Volume Per Well	Total in Master mix
rAPid Alkaline Phosphatase (10 U/ul)	1 ul	100 ul
rAPid Alkaline Phosphatase Buffer or 10x SAP Buffer	1 ul	100 ul
Exonuclease I (20U/ul)	0.05 ul	5 ul
Sterile Water	7.95 ul	795 ul
Total	**10 ul**	**-**

In each respective well, 10 ul of the PCR product and 10 ul of the master mix were added into the 96 Well .2mL PCR plate (GeneMate®), resulting in a total volume of 20 ul in each well. The PCR plate was then processed under the following Exo-SAPed conditions of 35°C for 45 minutes (Incubation) followed by 85°C for 15 minutes (Heat-kill).

2. Sequencing

Table 4. Sanger Sequencing Master Mix

Materials	Volume Per Well	Total in Master mix
1 uM Sequencing Primer	2.5 ul	250 ul
5x BigDye Dilution Buffer	2 ul	200 ul
BigDye Mix	0.5 ul	50 ul
Total	**5 ul**	

A master mix of the above contents were prepared on ice. 5 ul of the Exo-SAPed processed DNA and 5 ul of the above master mix were placed into each respective well in the 96 well plate, resulting in a total of 10 ul in each well. The plate was then placed under the following cycling conditions; incubation at 96°C for 3 minutes, followed by 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. The last three steps were repeated for 24 more cycles.

3. Sequencing Cleanup

Table 5. Sequencing Clean-up Contents

Materials	Volume Per Test
Sequenced DNA	10 ul (already in the plate)
125 mM EDTA	5 ul
Absolute Ethanol	60 ul
Total	**75 ul**

The above contents were added to each well separately. The plates were sealed, inverted several times, and incubated in the dark for 15 minutes at room temperature. The plate was then

spun at 2500 g for 30 minutes at 4°C. Once spun, the plate was unsealed, covered with a folded paper towel, inverted and spun to up to 185g. 60 ul of 70% ethanol was then added to each well and the plate was resealed, and spun at 1650 g for 15 minutes at 4°C. Afterwards, the plate unsealed once again, covered with a folded paper towel, inverted and spun to up to 185g.The plate was then left at room temperature, unsealed for 20 minutes to air dry. The plate was wrapped in aluminum foil and transported to the core lab for further processing.

2.1.1.8 Statistical Analysis

All statistical analysis was performed using the following analysis software: Microsoft Excel, GraphPad Prism 6, and STATA Special Edition 14.0**.** To assess the relationships between the variables, data was separated and compared into the following populations for each SNP:

1. HIV^+ vs. HIV^-
2. HIV^+ with cognitive decline vs. HIV^- with cognitive decline
3. HIV^+ without cognitive decline vs. HIV^- without cognitive decline
4. Cognitive decline vs. without cognitive decline

The degree of variation between each population was summarized graphically (Prism) and detailed in tables (Excel). Two sample binomial inference was concluded using odds ratio, risk ratio, and relative risk as statistical summaries; and p-value was determined using 2-sided Fisher's exact test, statistical significance was set at $p < 0.05$ (STATA).

2.1.2 Microbial Composition

2.1.2.1 Sample Collection

All stool samples were collected from previously registered MACS study subjects by outreach workers. The outreach workers meet up with subjects at a variety of community locations in the Pittsburgh area between April 1984- March 1985. Stool samples collected during this timeframe were stored at -20C prior to processing in 2016. **Table 13. List of Subject for Stool Samples**

2.1.2.2 DNA Extraction

DNA was purified and isolated using the MO BIO Laboratories, Inc. PowerFecal® DNA Isolation kit. The process involved 7 steps: preparation, cell lysis, inhibitor removal, DNA binding, washing, and eluting.

Preparation: A maximum amount of 0.25 grams of stool from each sample were placed into dry bead tubes along with 750 ul of Bead solution. The contents were gently vortexed to disband and separate stool particles. 60 ul of solution C1, a detergent containing disruption agents; was added to the tubes, manually mixed, and heat incubated for 10 minutes at 65C. This process prepares the samples for cell lysis by increasing the reaction rate between the lysis buffer and the sample products/ microbial cells. The tubes were then securely placed horizontally on the MO BIO Vortex Adapter and vortexed at maximum speed for 10 minutes. Samples were spun in a tube centrifuge for 1 minute at 13,000x g.

Cell Lysis and Inhibitor Removal: Supernatants from the dry bead tubes were transferred into clean 2 mL collection tubes. 250 ul of Inhibitor Removal Technology® or Solution C2 was placed into each tube. C2 solution removes inhibitory substances (cellular debris,

polysaccharides, etc.) from the stool samples that may interfere with downstream DNA applications amplification and sequencing processing. Tubes were spun at 13,000 x g for 1 minute. 600 ul of supernatant was transferred to a clean 2ml collection tube. 200 ul of solution C3 was added to each tube, briefly vortexed, then incubated at 4C for 5 minutes. Samples are then spun at 13,000 x g for 1 minute. 750 ul of the supernatant were transferred into clean 2 ml collection tubes.

DNA Binding: 1200 ul of solution 4, a high concentration salt solution that allows DNA to bind; was placed into each tube, then vortexed for 5 minutes. 650 ul of supernatant was then placed into Spin filter tubes, spun for 1 minute at 13,000x g. Flow through was discarded and the method was repeated until all supernatant underwent this process (a total of three time). The high salt solution allows for the DNA to bind to the silica filter device. Where DNA binds to the filter, contaminants pass through.

Washing: 500 ul of solution C5, an ethanol based wash solution; were placed into each to thoroughly clean bound DNA on the silica filter. Contents were spun for 1 minute at 13,000 x g and the flow through was discarded. Samples were spun again for 1 minute at 13,000 x g, allowing for the removal of excess wash solution.

Eluting: The spin filters were then transferred into clean 2ml collection tubes and 100 ul of solution C6, a sterile elution buffer; were directly added to the filter membrane. Tubes were spun at 13,000 x g for 1 minute and the spin filter basket discarded. Extracted DNA were stored at -20C until PCR amplification and 16s RNA sequencing were performed. DNA yields were monitored via gel electrophoresis.

2.1.2.3 Selection of Primers

Two forward and reverse 16S rDNA primers were chosen based on previous literature evaluations (Klindworth, 2013; Nossa, 2010). The following primers Klindworth-2013-341F, 5'CCT ACG GGN GGC WGC AG-3 and Klindworth-2013-785R 5' GAC TAC HVG GGT ATC TAA TCC-3; and Nossa-2010-803R 5' CTA CCR GGG TAT CTA ATC C-3 and Nossa-2010-347F GGA GGC AGC AGT RRG GAA T-3 were used in the PCR conditions as described in **Table 15. PCR Primers**. These primers include degenerate bases at many positions, in order to provide primers that will amplify the 16S region in as broad a range of bacterial taxa as possible.

2.1.2.4 Amplification

DNA samples underwent PCR processing to confirm the presence of bacterial genomic DNA. All PCR preparations were completed on ice, gently mixed, quickly spun, and then placed in Mastercycler® Gradient (Eppendorf) to complete the following program: Initial denaturation was set for 95°C for 5 minutes, followed by 25 cycles of denaturation at 95°C for 40 seconds, annealing at 55°C for 2 minutes, and at 72°C for 1 minute. This was followed by a final elongation at 72°C for 7 minutes**.**

Table 6. PCR Material

MATERIALS	PER SAMPLE
5 ug/ml of DNA sample (previously diluted)	2 ul
10x Buffer	2.5 ul
25 mM $MgCl_2$	1.5 ul
25 mM dNTPs	0.2 ul
10 mM Primers	0.4 ul
Taq Polymerase	0.2 ul
BSA	1.25 ul
DiH_2O	18.2 ul
TOTAL	25 ul

2.1.2.5 Gel Electrophoresis

DNA yields were confirmed via agarose gel electrophoresis. All PCR products were placed into a 2% gel that was prepared in a mixture consisting of 2 grams of GenePure LE Quick Dissolve Agarose powder (500g-ISC BioExpress) and 100ml of 0.5x TBE buffer. The mix was placed into a microwave and heated for a maximum of 3 minutes; this allowed the the agarose to thoroughly dissolve within the buffer. 10 ul of fluorescent dye (10,000x in water- Phenix Research GelRed) was placed into the gel solution. The gel solution was then placed aside to cool, then poured into a cast with pre-set combs and allowed to set into a gel. Once set, the gel was placed into the Gel X_L Ultra V-2 electrophoresis tank (Labnet International, Inc.). The gel was completely submersed within 0.5x TBE buffer. The samples were then prepared for loading with the following 1:10 concentration:

Table 7. Agarose Gel

	Amount per Sample
10X LOADING DYE (w/ xylene cyanol and bromophenol blue)	1 ul
DNA SAMPLE (PCR Product)	2 ul
DIONIZED WATER	7 ul

The mixture was vortexed and spun. 5 ul of the above mix was placed into each respective well; one well was designated for the ladder PHIX174 DNA/*Hae*III Digest Marker (50ng/ul- Thermo Scientific). The gel electrophoresis was set for 30 minutes at a voltage of 100v.

Analysis of the PCR products in the stained DNA gel were visualized and captured on a UV transilluminator, Red™ Imaging System (Alpha Innotech).

3.0 RESULTS

3.1 SNPS INVOLVED IN FOLATE METABOLISM AND DNA METHYLATION

The raw data from the allelic discrimination analysis was converted into frequency tables using Microsoft Excel and that data was then plugged into the GraphPad Prism 6 software to generate the following graphs. Each SNP was analyzed for its association to cognitive decline and compared within the following groups:

- **HIV^- individuals vs. HIV^+ individuals**
- **HIV^- individuals with Cognitive Decline vs. HIV^+ individuals with Cognitive Decline**
- **HIV^- individuals without Cognitive Decline vs. HIV^+ individuals without Cognitive Decline**
- **Individuals with Cognitive Decline vs. Individuals without Cognitive Decline**

3.1.1 Allelic Discrimination

The data underwent an auto-scaling process in which genotyping software automatically determined the results of the assay. Allele specific TaqMan probes with linked reporter dyes, FAM™/SYBR® Green linked to allele 2, and VIC®/JOE™ linked to allele 1; detected alleles specific to the polymorphism of interest. The data was generated into allele discriminating plots for each SNP.

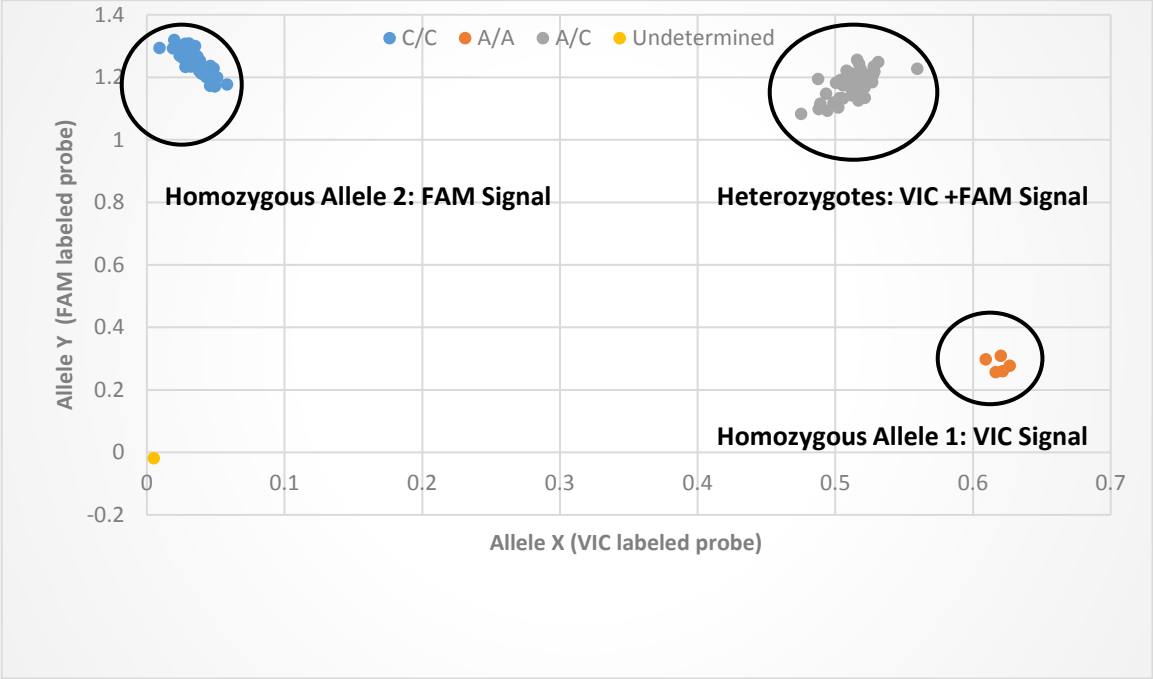

Figure 4. Allelic Discrimination of SNP rs4925- GST02/MIR4482-1

Majority of the raw data measuring location and intensity of each signal, were able to form 3 distinct clusters. However, for one SNP gene, rs1801133; the cluster formation was less distinct resulting in a muddled allelic discrimination plot that prove to be difficult to delineate any cluster groups.

We proceeded with Sanger Sequencing to further define the allele pairing sequences at a nucleotide baes level, which allowed use to confirm the genotypes and generate the following graph.

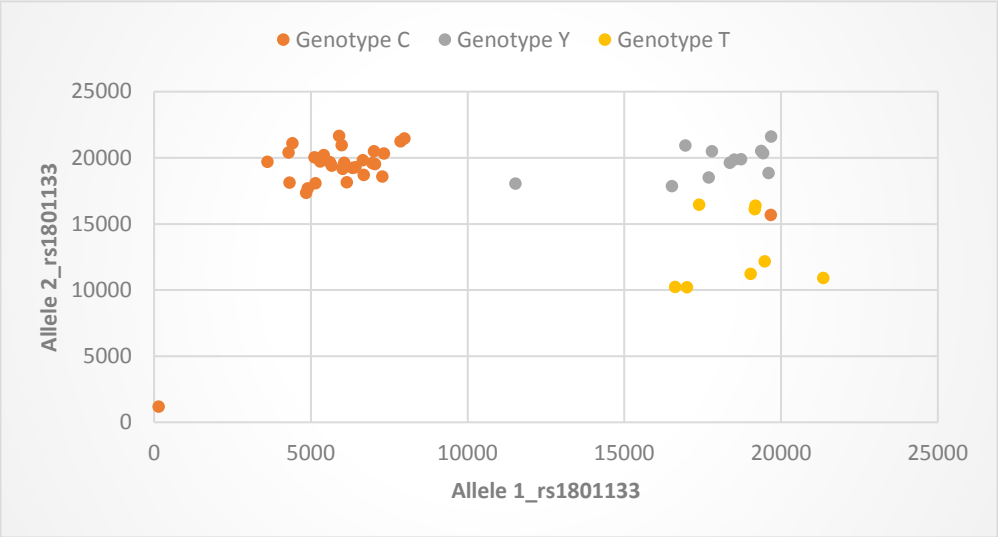

Figure 5 Allele Discrimination Plot rs1801133- MTHFR

In conclusion, there was no significant difference in grouping between the groups; however majority of the genes in our assay panel were able to form three distinct cluster groups of allelic variants in homozygous and heterozygous form. To further asses the results of our data, allele frequencies and associated risk summaries were graphed and calculated to further delineate any association to HIV infection and cognitive decline in each group for each SNP.

3.1.2 Risk Association

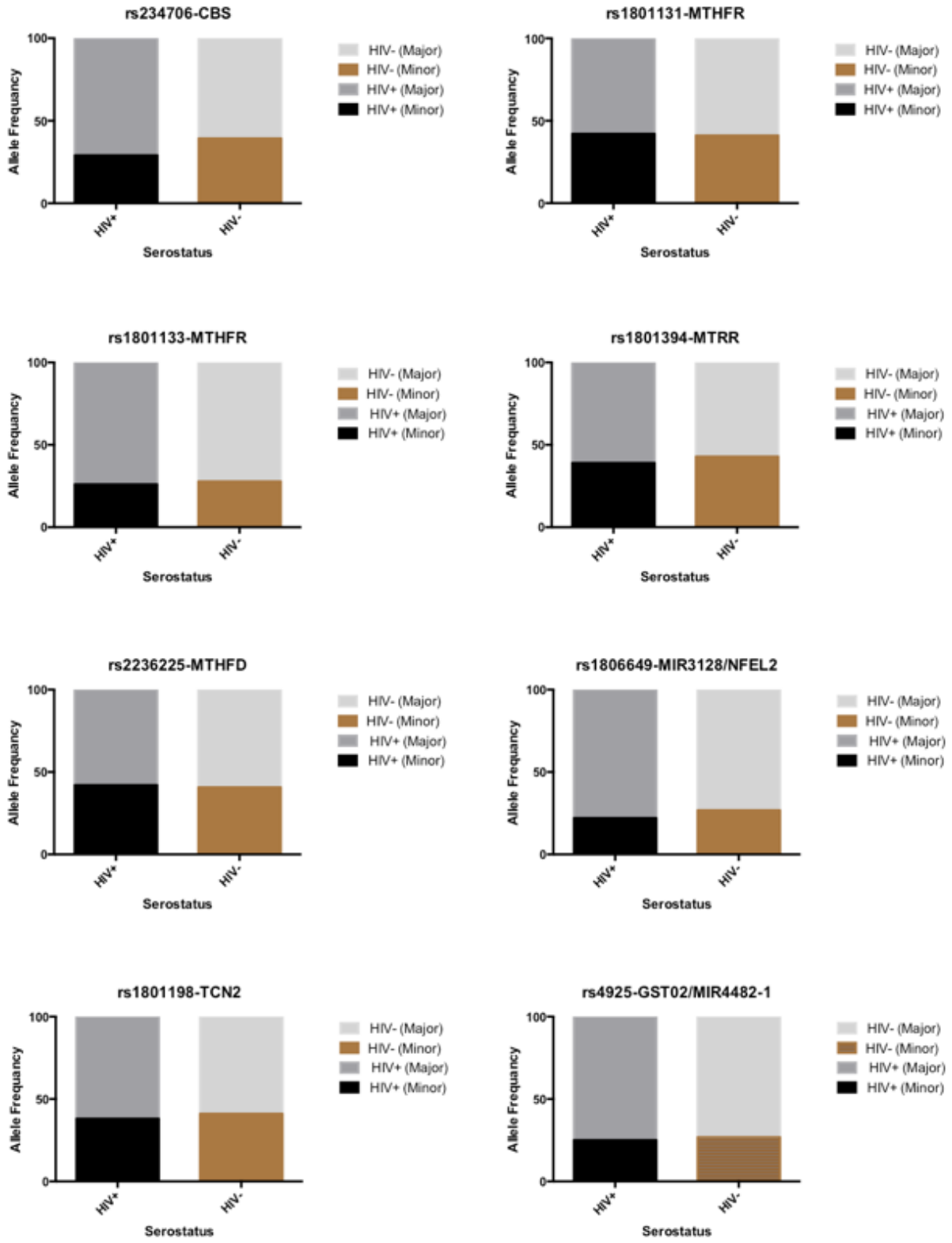

Figure 6. Frequency of SNP alleles in HIV^{+} individuals versus HIV^{-} individuals absent of cognitive condition

Table 8. SNP Association Results: HIV$^+$ versus HIV$^-$

SNPs	Minor Allele	MAF (HIV$^+$)	MAF (HIV$^-$)	OR (95% CI)	P-value
rs234706	A	0.38	0.27	1.58 (.795-3.15)	0.2150
rs1801131	G	0.55	0.20	0.962 (.495-1.87)	1.000
rs1801133	A	0.34	0.18	1.09 (.518-2.31)	0.8497
rs1801394	G	0.51	0.29	1.17 (.602-2.28)	0.734
rs2236225	C	0.55	0.27	.917 (.466-1.80)	0.865
rs1806649	T	0.29	0.18	1.30 (.607-2.77)	0.5579
rs1801198	C	0.50	0.28	1.14 (.582-2.22)	0.7345
rs4925	A	0.33	0.18	1.10 (.520-2.32)	0.8495

Abbreviations: SNPs = Single Nucleotide Polymorphisms, MAF = Minor Allele Frequency in cases and controls, OR = Odds Ratio. Cases are subjects who are HIV$^+$ at time of collection, controls are subjects who are HIV$^-$ at the time of collection.

Table 8 depicts the statistical characteristics of single-nucleotide polymorphisms in folate genes amongst HIV+ (cases) and HIV- (control) subjects. We began by comparing the Minor Allele Frequencies (MAF) for the total case and control populations from each SNP and determined their risk of association to cognitive decline. In the graphical description, there is an equal distribution of data between each group within the HIV+ and HIV- population. For both populations (HIV+/HIV-) there is a higher frequency of major alleles being expressed and an equally lower amount of minor allele frequency being expressed between each group. There is one exception in the CBS gene (rs234706), the HIV- population seems to express a slightly higher frequency than the HIV+ population, however the frequency is not greater than the

expression level of major allele frequencies. Six out of eight SNPs expressed an Odds Ratio (OR) >1: (rs234706, rs1801133, rs1801394, rs1806649, rs1801198, rs4925); suggesting that for these genes, an individual expressing minor alleles are more at risk of the adverse outcome of cognitive decline. Two SNPs expressed OR < 1: (rs1801131 and rs2236225), suggesting that an individual expressing the minor alleles for these genes (*MTHFR, MTHFD1*) are at less risk of cognitive decline. However, all SNPs comparing HIV^+ vs. HIV^- groups expressed p-values > 0.05; concluding that none of the associations were statistically significant.

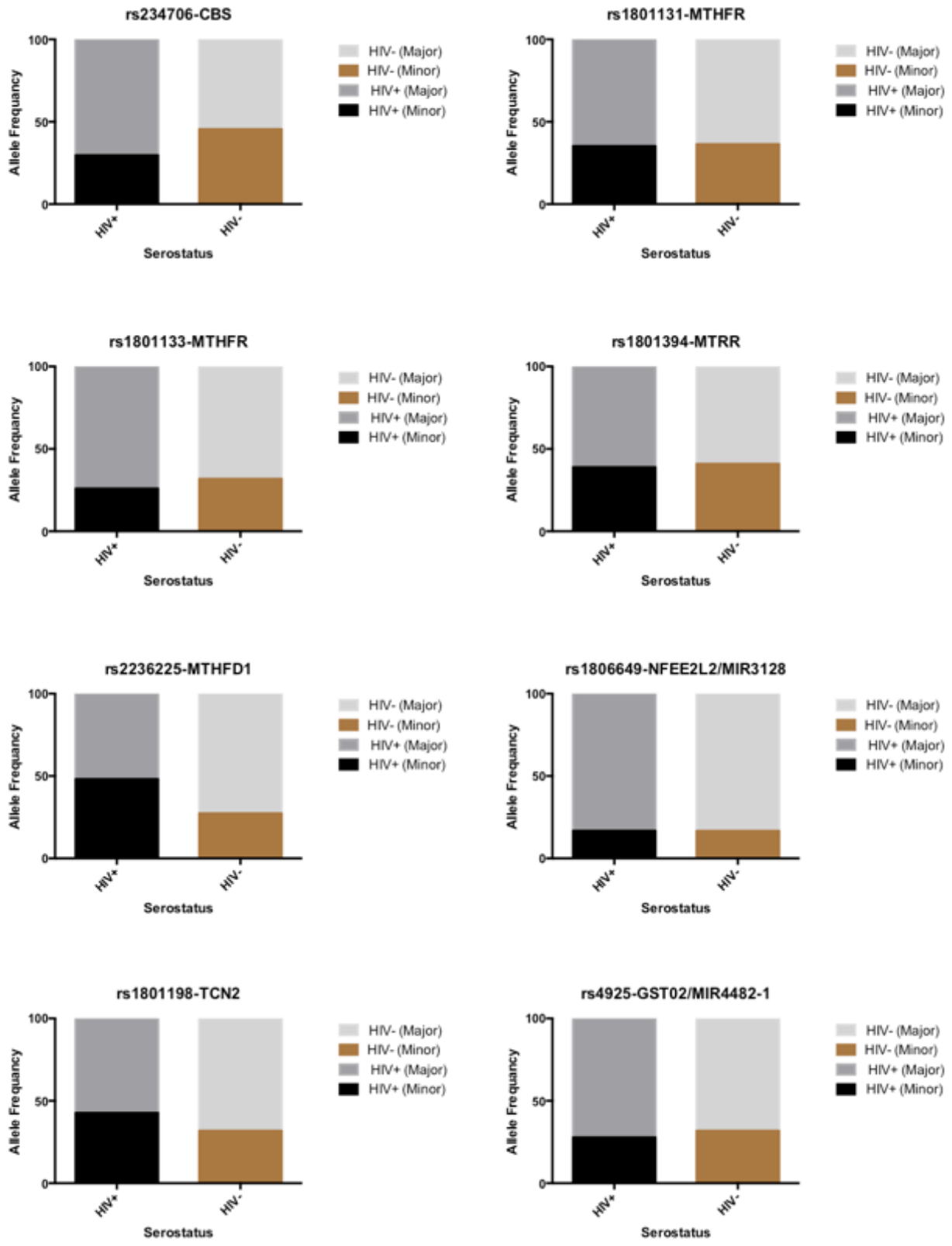

Figure 7. SNP Allele Frequency distributions in HIV- individuals with Cognitive Decline versus HIV+ individuals with Cognitive Decline

Table 9. SNP Association Results: HIV^+ versus HIV^- with Cognitive Decline

SNPs	Minor Allele	MAF (HIV^+/CD)	MAF (HIV^-/CD)	OR (95% CI)	P-value
rs234706	A	0.30	0.45	1.98 (.713-5.59)	0.1969
rs1801131	G	0.35	0.36	1.05 (.372-2.98)	1.000
rs1801133	A	0.26	0.32	0.667 (.214-2.07)	0.577
rs1801394	G	0.39	0.41	1.088 (.393-3.01)	1.00
rs2236225	C	0.48	0.27	0.403 (.139-1.178)	0.126
rs1806649	T	0.17	0.23	1.47 (.429-5.04)	0.5308
rs1801198	C	0.43	0.32	0.629 (.220-1.797)	0.4456
rs4925	A	0.28	0.32	1.21 (.411-3.584)	0.7834

Abbreviations: SNPs = Single Nucleotide Polymorphisms, MAF = Minor Allele Frequency in cases and controls, OR = Odds Ratio, CD = Cognitive Decline. Cases are subjects who are HIV^+ at time of collection, controls are subjects who are HIV^- at the time of collection, both groups exhibit cognitive decline.

Table 9 depicts the statistical characteristics of SNPs in folate genes amongst the HIV^+ with Cognitive Decline and the HIV^- with Cognitive Decline. To further delineate if there is an association amongst the HIV+ and HIV- populations, we sought to compare the two groups with the condition of Cognitive Decline. There is more variation of data distribution within these groups. In the following SNPs: rs1801131, rs1801394, rs1806649, and rs4925; the distribution of data is similar to what was seen in the previous graphs. There is equal distribution of minor and major alleles between the two groups were there is greater expression of the major allele within these SNPs. rs2234225 and rs1801198 graphically show a distribution pattern of a higher frequency of minor alleles being expressed in HIV+ population and a higher frequency of major

alleles being expressed in HIV- population. For the SNPs rs234706 and rs1801133, majority of the HIV+ population is expressed a higher frequency of the major alleles and the HIV- population expressed slightly higher frequency of the minor allele. Five out of eight SNPs expressed an OR > 1: (rs234706, rs1801131, 1801394, 180649, rs4925); suggesting that for those genes, individuals expressing the minor alleles are at higher risk of cognitive decline than those expressing the major alleles. Three SNPs expressed OR < 1: (rs1801133, rs2236225, 1801198), suggesting that in relation to those genes, individuals expressing the minor alleles are at less risk of cognitive decline. However, all SNPs comparing HIV^+ with Cognitive Decline vs. HIV^- with Cognitive Decline expressed p-values > 0.05; concluding that none of the associations were statistically significant.

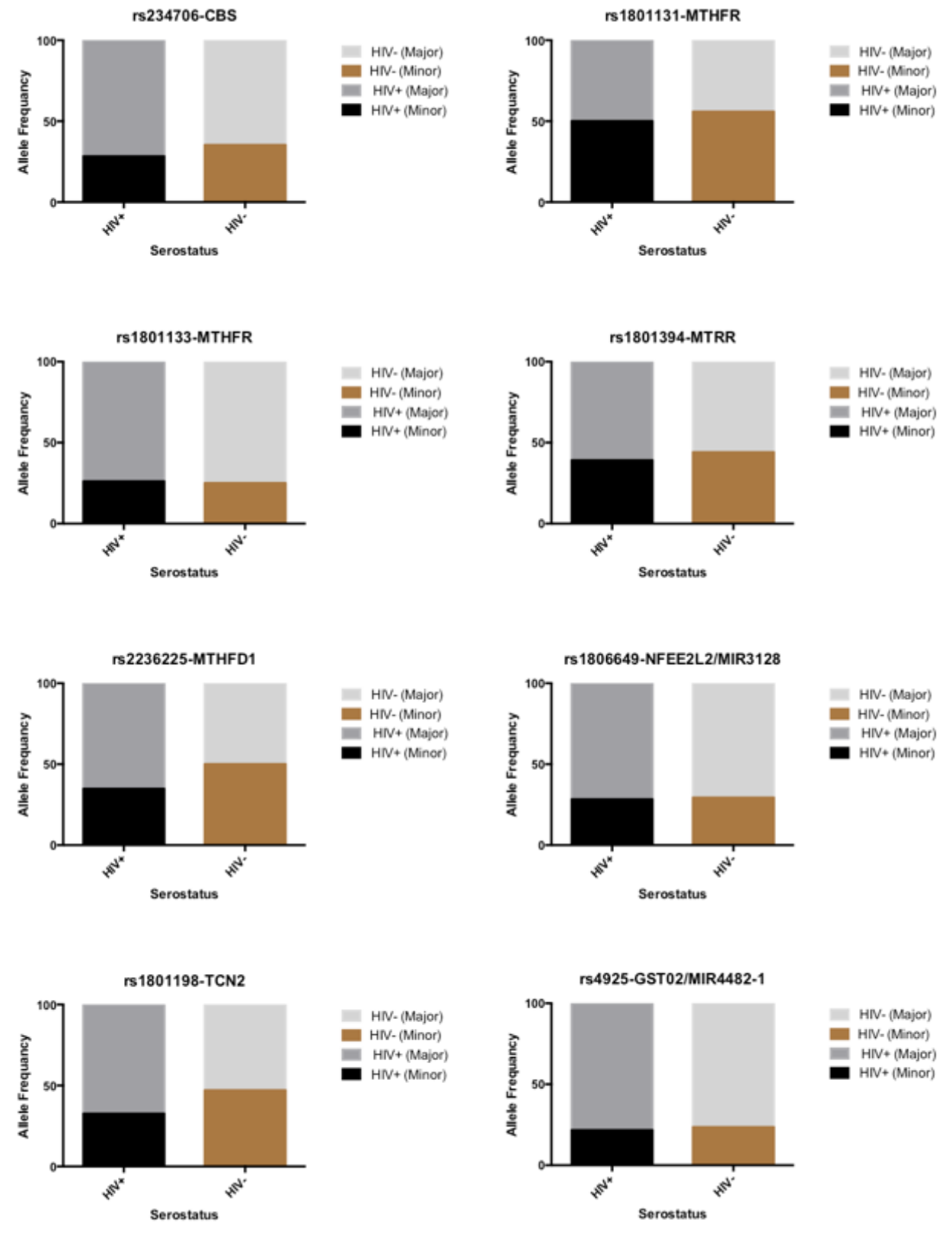

Figure 8. SNP Allele Frequencies of HIV$^-$ individuals without Cognitive Decline versus HIV+ individuals without Cognitive Decline

Table 10. SNP Association Results: HIV$^+$ versus HIV$^-$ without Cognitive Decline

SNPs	Minor Allele	MAF (HIV$^+$/No CD)	MAF (HIV$^-$/No CD)	OR (95% CI)	P-value
rs234706	A	0.28	0.33	1.384 (.531-3.60)	0.6265
rs1801131	G	0.50	0.22	1 (1-1)	1.000
rs1801133	A	0.26	0.22	.944 (.333-2.68)	1.000
rs1801394	G	0.39	0.42	1.228 (.497-3.03)	0.8186
rs2236225	C	0.35	0.44	1.75 (.691-4.429)	0.2505
rs1806649	T	0.28	0.28	1.058 (.395-2.83)	1.00
rs1801198	C	0.33	0.44	1.837 (.736-4.58)	0.247
rs4925	A	0.22	0.22	1.108 (/383-3.21)	1.000

Abbreviations: SNPs = Single Nucleotide Polymorphisms, MAF = Minor Allele Frequency in cases and controls, OR = Odds Ratio, CD = Cognitive Decline. Cases are subjects who are HIV$^+$ at time of collection, controls are subjects who are HIV$^-$ at the time of collection, neither groups exhibit cognitive decline.

Table 10 depicts the statistical characteristics of folate genes amongst the HIV$^+$ without Cognitive Decline and the HIV- without Cognitive Decline. SNPs rs1806649, rs4925, rs1801133, and rs1801394 show an equal distribution of major and minor alleles amongst the HIV+ and the HIV- population where there expressing higher frequencies of the major alleles. rs234706, rs2236225, and rs1801198 are expressing higher frequencies of major alleles in the HIV+ population versus a slightly higher frequency of minor alleles in the HIV- population. Rs1801131 is expressing a unique variation of distribution where the HIV+ population is expressing a higher frequency of minor alleles versus a higher frequency of major alleles in the HIV- population. Six out of eight SNPs expressed an OR > 1: (rs2347, rs1801394, rs2236225, rs1806649, 1801198, rs4925); suggesting that for those genes**,** individuals expressing minor

alleles are more at risk of cognitive decline. One SNP expressed OR < 1: (1801133), suggesting that in relation to those genes, individuals expressing minor alleles are at less risk of cognitive decline. However, One SNP had an OR equal to 1: (rs1801131), suggesting that there is no significant difference between the two groups. However, all SNPs comparing HIV^+ without Cognitive Decline vs. HIV^- without Cognitive Decline expressed p-values > 0.05; concluding that none of the associations were statistically significant.

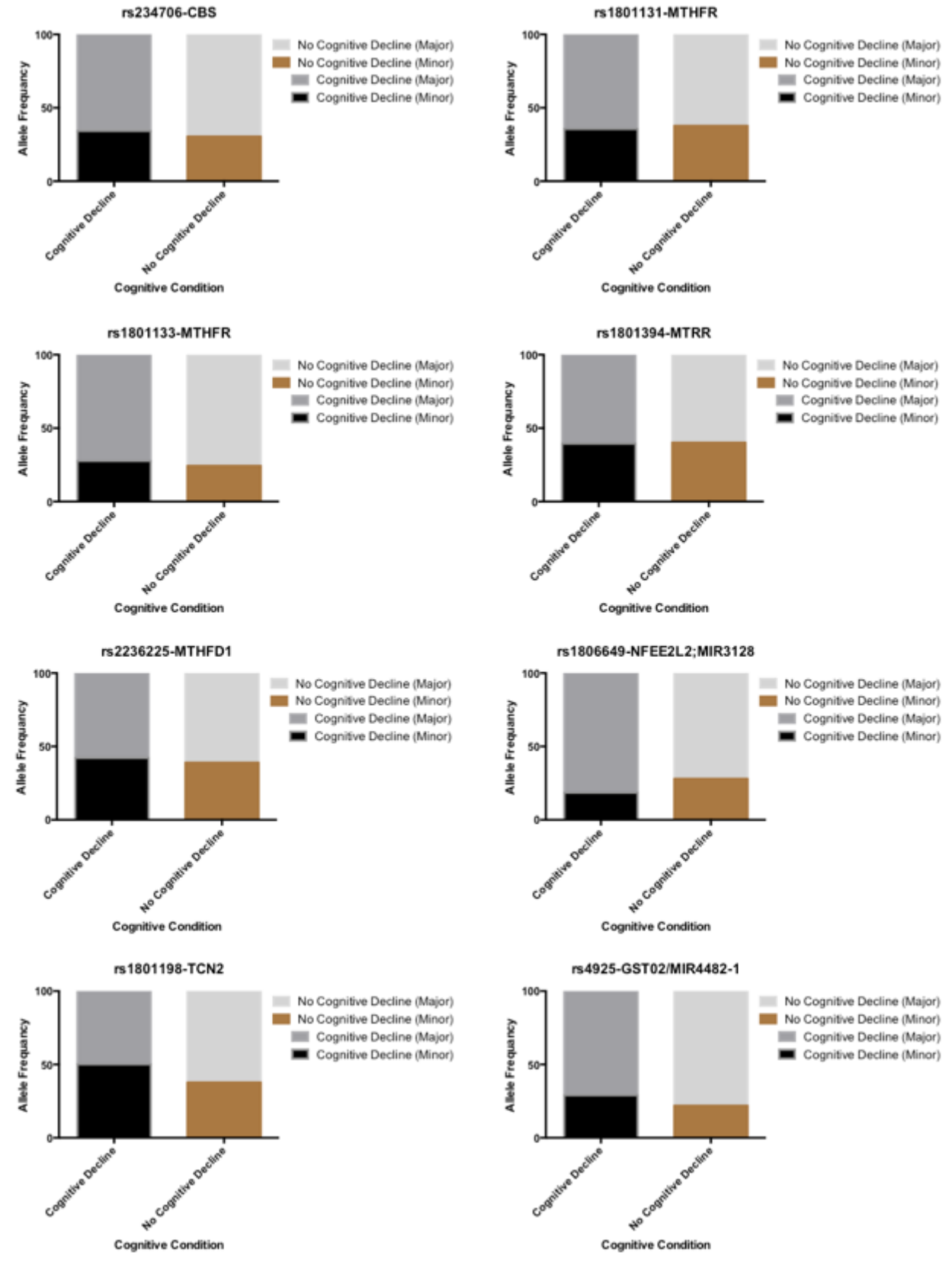

Figure 9. Frequency of SNP Alleles in individuals with Cognitive Decline versus No Cognitive Decline

Table 11. Single SNP Association Results: Cognitive Decline versus No Cognitive Decline

SNPs	Minor Allele	MAF-CD	MAF-No CD	OR (95% CI)	P-value
rs234706	A	0.34	0.30	.874 (447-1.71)	0.7347
rs1801131	G	0.36	0.38	1.81 (.948-3.48)	0.1008
rs1801133	A	0.28	0.24	.903 (.440-1.85)	0.8560
rs1801394	G	0.39	0.40	1.08 (.556-2.05)	0.8711
rs2236225	C	0.42	0.39	1 (-)	1.000
rs1806649	T	0.18	0.28	1.79 (.842-3.79)	0.1376
rs1801198	C	0.39	0.38	.702 (.372-1.32)	0.3348
rs4925	A	0.29	0.22	.712 (.346-1.47)	0.3667

Abbreviations: SNPs = Single Nucleotide Polymorphisms, MAF = Minor Allele Frequency in cases and controls, OR = Odds Ratio, CD = Cognitive Decline. Cases are subjects who experience cognitive decline, controls are subjects who do not exhibit cognitive decline.

Table 11 depicts the statistical characteristics of folate genes amongst the Cognitive Decline group and the group Without Cognitive Decline. For majority of the SNPs compared within this group, the distribution pattern is similar between the groups (cognitive decline vs. without cognitive decline) though the levels vary between the SNPS. Both groups are expressing higher allele frequencies of the major alleles. In SNP rs1806649, the population without cognitive decline is expressing a slightly higher frequency of minor alleles than the population with cognitive decline, but overall the major allele frequencies are expressed at a higher level. In the SNP rs4925, the reverse is seen, the population with cognitive decline is expressing a slightly higher level of major alleles than the population without cognitive decline, but overall the the

major allele frequencies are expressed at a higher level. Three out of eight SNPs expressed an OR >1: (rs1801131, rs1801394, rs1806649); suggesting that for those genes, individuals expressing minor alleles are more at risk of becoming HIV^+ or developing cognitive decline. Four SNPs expressed OR < 1: (rs2347006, rs1801133, rs1801198, rs9425), suggesting that in relation to those genes, individuals expressing minor alleles are less at risk of becoming HIV^+ or developing cognitive decline. However, One SNP had an OR equal to 1: (rs223225), suggesting that there is no significant difference between the two groups. However, all SNPs comparing Cognitive Decline vs. No Cognitive Decline groups expressed p-values > 0.05; concluding that none of the associations were statistically significant.

In conclusion, we do not reject that null hypothesis of the odds ratio equaling 1 for the following comparisons: HIV^- *vs* HIV^+; HIV+ with cognitive *vs* HIV^- with cognitive decline; HIV+ without cognitive decline *vs* HIV^- without cognitive decline; and cognitive decline *vs* no cognitive decline regardless of serostatus, based on the two-sided Fischer's exact test at level 0.05. We have do not have sufficient evidence to conclude that for the following genes: (*CBS, MTHFR, MTRR/FASTKD3, MIR3128/NFEE2L2, TCN2/PES1, GST02/MIR4482-1, MTHFD1*), germline genetic polymorphisms are associated with risk of either being HIV^+ or developing cognitive decline (Rosner, 2011).

3.2 MICROBIAL COMPOSITION

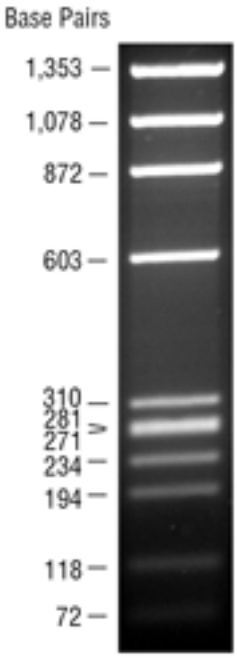

Figure 10. DNA Ladder Map (Scientific, 2016)

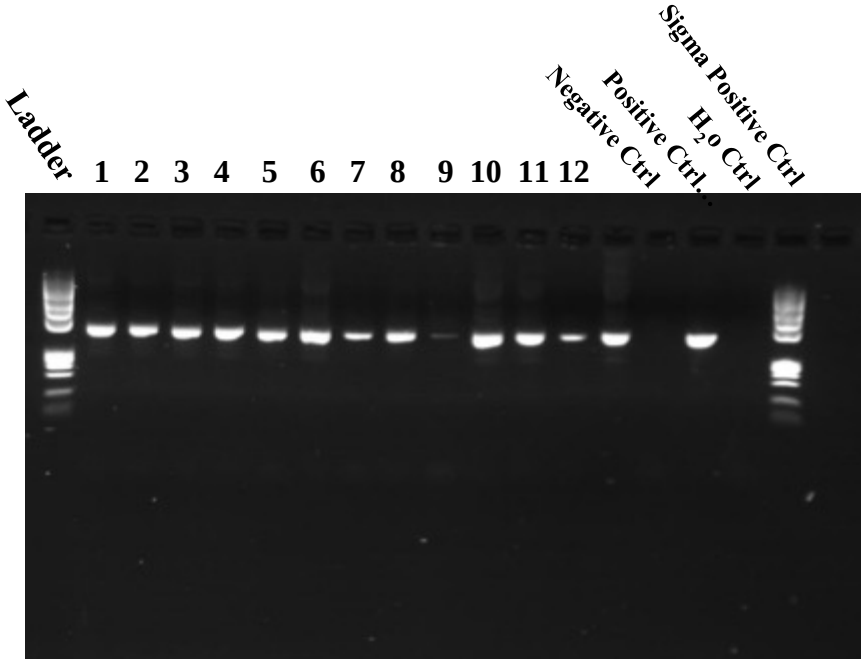

Figure 11 Gel Electrophoresis of Isolated Stool DNA

Figure 9: Results of PCR product. Gel electrophoresis was conducted on amplified DNA samples to ensure the presence of bacterial genomic DNA. The primers used are predicted to give a PCR product with an estimated size of 444 bp, which can be seen in the above gel electrophoresis image, suggesting that the archival fecal samples we processed retain microbiome genetic material that is suitable for 16S RNA sequencing analysis.

4.0 DISCUSSION

HAND and its neuropathological conditions that attribute to neurocognitive decline and accelerated aging remain to be imminent ailments that afflict long term infected individuals. The limitations and subjectivity within the current methods of analysis leaves much margin for error impacting diagnosis, treatment, and therapy; all of which aids in the morbidity and decreased quality of life seen within these infected individuals. DNA methylation has so many implications in healthy development and cellular growth; and aberrations in the pathways that mediate DNA methylation also heavily impact disease development and cellular dysfunction. Identifying biomarkers associated with cognitive decline could subsidize the burden of disease within the infected population and also assist in early diagnosis for those genetically predisposed within the uninfected population.

The gut microbiota facilitates so many facets within an organism; digestion, priming of the immune system, maintaining immune function, and symbiosis. All efforts influence the host's behavior and the CNS activity. Alterations to that environment leads to dysfunction and other systemic immunodeficiencies. Accepting that the gut microbiota is an important facet in immunity and health makes it all the more imperative to understanding how diversity in the gut microbiome can affect health and aging. Identifying microbial factors could provide better options for regulating chronic HIV infection and neuropathology**.** The overall goal is to gain better insight of the underlying causes of age related morbidities and neurocognitive disorders to

aid in early diagnosis, provide a more refine form of treatment, and to grasp a better understanding of the mechanisms in which neurocognitive decline is occurring. This was the purpose of all our efforts in which we sought to identify key factors by monitoring genetically inherited single nucleotide polymorphisms of folate genes and compositional alterations in the microbiota of individuals.

In our first project monitoring SNPs involved in folate metabolism and DNA methylation; we compared the following sample groups: HIV^{-}/cognitive $decline^{-}$, HIV^{-}/cognitive $decline^{+}$, HIV^{+}/$HAND^{-}$, HIV^{+}/$HAND^{+}$. We did not see any significant associations of single nucleotide polymorphisms and the occurrence of cognitive decline. Though the data did not produce any significant inference, the variable distribution seen within the graphs of certain SNPs suggests that there could be some minor differences between populations (HIV+/: rs1801131, rs1801394, rs1801133, rs2236225, rs1806649, rs1801198, rs4925; HIV+/- w/ cognitive decline: rs234706, rs2236225, rs1801198; and HIV+/- No CD: rs1801131). Under the null hypothesis, there is no association to cognitive decline in relation to polymorphism in the folate genes amongst the various groups we observed. Based on our statistical summary, we were not able to reject the null hypothesis. Unfortunately, our sample size (86 subjects) may not have been large enough to notice any significant differences. Increasing the sample size within each group would increase the power of our test, decrease variations, and narrow the confidence interval; all of which provide more precise information about the parameter. Power is a statistical measure that asses a tests ability to accurately detect a result, if the result did in fact occur. Power, also referred to as sensitivity, allows us to compare the probabilities of success within the two populations we are are observing and is heavily influenced by effect size (Rosner, 2011).

Now when the sample size increases the power increases and that then decreases the chance of the test picking up false negatives or type II error.

Future directions would include looking at other genes associated with the regulation of the complement cascade, clearance of immune complexes, and immune response. But even if we decided to look at these candidate genes that have been shown to contribute to a form of dementia, our current sample size would not allow us to infer any significant association between the groups. Another hurdle would be attaining subjects of a certain group. Acquiring more subjects for the HIV^+/ $HAND^+$ group would be difficult, as these subjects make up a small portion of the infected population.

As shown previously in the results, we were able to determine that there was bacterial DNA within the samples that we processed. The samples have been sent to the University of Pittsburgh Genome Research Core to undergo 16S RNA characterization by amplicon sequencing on the Ion Torrent platform (Life Technologies). Once the samples are sent off to the core, they begin profiling the bacterial 16S rRNA in the sample. This is done through the use of primers designed to amplify hypervariable regions in the bacterial 16S rRNA gene. A multiplex PCR will target these regions and produce amplicon products of varying size. Those amplicon products are then used to create libraries and templates. The samples are sequenced through an Ion PGM semiconductor sequencer. This is a next generation process that detects released hydrogen ions during DNA synthesis and converts the data into a digital signal (Scientific, ThermoFisher, 2016). Once complete a bioinformatic software will automatically upload the output data and classify the reads using 1 of 3 reference libraries. This process allows us to identify the complex microbial composition within each stool sample and differentiate what is

observed by taxonomy and phylogeny. At the time in which this thesis was written, we have not received the output data report.

Once we receive the data we can use various diversity indexes to measures the level of species variation within the gut of our sample subjects. The Shannon entropy and Simpson diversity index would allow us to determine the species richness within the gut (alpha diversity) and determine the extent in which certain species were gained or lost within the gut as well (beta diversity). Richness is defined as the amount of species found within the site that are genetically related and evenness measure the abundance of species in proportion to the richness of a species within the site (University of Idaho, 2009).

Some possible confounding factors that may affect what we do see within in our samples is the process in which the samples were collected and the amount of time the samples spent in storage. As stated previously, the samples were collected in a variety of community locations, some of which were non-clinical settings. The process in which the stool samples were collected may have introduced some environmental contamination that may skew the biodiversity results that we might find. Also the stool samples were collected well over 30 years ago; this introduces several different extraneous circumstances that may impact our data results. Years in the freezer may have caused some deterioration to occur, degrading certain species of bacteria that could not withstand the long term conditions of storage while others remained intact for years. This random process compromises the integrity of the samples in a way that leads to bias produced results. It is for this reason we checked for DNA products prior to shipping off our samples to the core lab in an effort to compensate for degradation and any compromise that may have occurred throughout the years in storage.

The normal gut microbiome is made up of enterotypes including the following species: *Bacteriodes, Prevotella, and Ruminococcus*. These species work together to maintain homeostasis and symobiosis within the gut envrionment. However during HIV infection, a series of alterations in the compsition and other facets occur. In a study conducted by Zevin et a, (2016); they determine which microorgnaisim were enriched while others decreased in HIV infected individuals. The following set of bacteria increased in compositon in HIV infected indiduals: *Provotella, Pseudomonas, Desulfovibrio, Acinoetobacter. Campylobacter, Escherichia, Ruminococcus.* While the following set of bacteria depleted with the microbiome in HIV infected individual: *Bacteroides, Lactobacillus, Bifidobacterium, Eubacterium, Coprococcus, Blautia, Ruminococcus* (Zevin, McKinnon, Burgerner, & Klatt, 2016). For certain diet and the condition of the immune system "structures" the gut microbiota. HIV is known to negatively impact the cellular and molecular processes of both the innate and adaptive immune system. Both these systems are essential in shaping the composition of the intestinal bacteria; the difference in microbial composition may impact how the immune system responds to HIV and other infections. So gaining a better perspective on these factors could help facilitate treatment for gut-associated diseases and its effect on HIV pathogenesis.

With all the progress made in research and treatment development HIV has transformed into a chronic disease in which quality of life could be stable if active on antiretroviral regimen. However, even with all that, the attributing mechanism of neuropathogenesis and HAND has yet to be distinctively identified. Because of that finding biomarkers like the ones we looked at SNPs, components of the microbiome, etc., or in other forms is an important public health outcome that we should continue to strive an answer for. These biomarkers could serve as early

indicators of not only HAND but other age- related cognitive disorders in both HIV infected individuals and uninfected individuals. within the infected population.

Overall, comparing and identifying significant genetic and microbial factors associated with HAND within the HIV-infected subpopulation, could subsequently resolve the burden of disease suffered within this subpopulation. Further investigation into how these factors may or may not contribute to disease development in each individual is of importance for both science and public health.

In future directions monitoring the difference in cellular immune response in relation to immune dysfunction and alterations in gut microbiota and asses how disease severity impacts gut microbiota composition can provide more insight to the level of influence the gut microbiota has on HIV pathogenesis.

APPENDIX A: SUPPLEMENTAL TABLES

Table 12. List of Subjects and Sero-status

ID	CONDITION	ID	CONDITION	ID	CONDITION
1	HIV-/Cognitive Decline- (M)	**33**	HIV+/Cognitive Decline+	**65**	HIV-/Cognitive Decline-
2	HIV-/Cognitive Decline- (M)	**34**	HIV+/Cognitive Decline+	**66**	HIV+/Cognitive Decline-
3	HIV-/Cognitive Decline- (M)	**35**	HIV+/Cognitive Decline+	**67**	HIV+/Cognitive Decline-
4	HIV-/Cognitive Decline-	**36**	HIV+/Cognitive Decline+	**68**	HIV+/Cognitive Decline-
5	HIV-/Cognitive Decline-	**37**	HIV+/Cognitive Decline+	**69**	HIV+/Cognitive Decline-
6	HIV-/Cognitive Decline- (M)	**38**	HIV+/Cognitive Decline+	**70**	HIV+/Cognitive Decline-
7	HIV-/Cognitive Decline- (M)	**39**	HIV+/Cognitive Decline+	**71**	HIV+/Cognitive Decline-
8	HIV-/Cognitive Decline- (M)	**40**	HIV+/Cognitive Decline+	**72**	HIV+/Cognitive Decline-
9	HIV-/Cognitive Decline- (M)	**41**	HIV+/Cognitive Decline+	**73**	HIV+/Cognitive Decline-
10	HIV-/Cognitive Decline- (M)	**42**	HIV+/Cognitive Decline+	**74**	HIV+/Cognitive Decline-
11	HIV-/Cognitive Decline+ (M)	**43**	HIV+/Cognitive Decline+	**75**	HIV-/Cognitive Decline+
12	HIV-/Cognitive Decline+ (M)	**44**	HIV+/Cognitive Decline+	**76**	HIV-/Cognitive Decline-
13	HIV-/Cognitive Decline+ (M)	**45**	-	**77**	HIV-/Cognitive Decline-
14	HIV-/Cognitive Decline+ (M)	**46**	-	**78**	HIV+/Cognitive Decline-
15	HIV-/Cognitive Decline+ (M)	**47**	-	**79**	HIV-/Cognitive Decline+
16	HIV+/Cognitive Decline- (M)	**48**	-	**80**	HIV-/Cognitive Decline-
17	HIV+/Cognitive Decline- (M)	**49**	-	**81**	HIV-/Cognitive Decline+
18	HIV+/Cognitive Decline- (M)	**50**	-	**82**	HIV+/Cognitive Decline-
19	HIV+/Cognitive Decline- (M)	**51**	HIV+/Cognitive Decline+	**83**	HIV-/Cognitive Decline+
20	HIV+/Cognitive Decline-	**52**	HIV+/Cognitive Decline+	**84**	HIV-/Cognitive Decline+
21	HIV+/Cognitive Decline- (M)	**53**	HIV+/Cognitive Decline+	**85**	HIV-/Cognitive Decline+
22	HIV+/Cognitive Decline- (M)	**54**	HIV+/Cognitive Decline+	**86**	HIV-/Cognitive Decline+
23	HIV+/Cognitive Decline- (M)	**55**	HIV+/Cognitive Decline+		
24	HIV+/Cognitive Decline- (M)	**56**	HIV-/Cognitive Decline-		
25	HIV+/Cognitive Decline+ (M)	**57**	HIV+/Cognitive Decline-		
26	HIV+/Cognitive Decline+ (M)	**58**	HIV+/Cognitive Decline+		
27	HIV+/Cognitive Decline+ (M)	**59**	HIV+/Cognitive Decline-		
28	HIV+/Cognitive Decline+	**60**	HIV+/Cognitive Decline-		
29	HIV+/Cognitive Decline+	**61**	HIV+/Cognitive Decline-		
30	HIV+/Cognitive Decline+	**62**	HIV+/Cognitive Decline-		
31	HIV+/Cognitive Decline+	**63**	HIV+/Cognitive Decline-		
32	HIV+/Cognitive Decline+	**64**	HIV-/Cognitive Decline-		

Table 13. List of Subject for Stool Samples

	Sero-Status	Date of Repeat
1	**HIV+**	**-**
2	**HIV-**	**-**
3	**HIV-**	**-**
4	**HIV-**	**4/17/84**
5	**HIV+**	**-**
6	**HIV+**	**-**
7	**HIV-**	**10/17/84**
8	**HIV+**	**-**
9	**HIV-**	**-**
10	**HIV-**	**-**
11	**HIV-/HIV+**	**-**
12	**HIV-**	**4/25/85**

Table 14. TaqMan SNP Genotyping Assay List

SNP ID	Gene	Location	Polymorphism
rs234706	CBS	Chr.21: 44485350	**A**/G
rs1801131	MTHFR	Chr.1: 11854476	**G**/T
rs1801133	MTHFR	Chr.1: 11856378	G/**A**
rs1801394	MTRR/FASTKD3	Chr.5: 7870973	A/**G**
rs236225	MTHFD1	Chr.6: 79115935	**C**/G
rs1806649	NFEE2L2;MIR3128	Chr.2: 178118152	C/**T**
rs1801198	TCN2;PES1	Chr.22: 31011610	**C**/G
rs4925	GST02/MIR4482-1	Chr.10: 106022789	**A**/C

Table 15. PCR Primers

Gene		Sequence (5'-3')
MTHFR C677T	Forward	TGA AGG AGA AGG TGT CTG GGG GA
	Reverse	AGG ACG GTG CGG TGA GAG TG
MTHFR C677T	Forward	TCT TCA TCC CTC GCC TTG AAC
	Reverse	AAG TGA TGC CCA TGT CGG TG
KLINDWORTH-2013-341F/785R	Forward	CCT ACG GGN GGC WGC AG
	Reverse	GAC TAC HVG GGT ATC TAA TCC
NOSSA-2010-347F/803R	Forward	GGA GGC AGC AGT RRG GAA T
	Reverse	CTA CCR GGG TAT CTA ATC C

APPENDIX B: SUPPLEMENTAL FIGURES

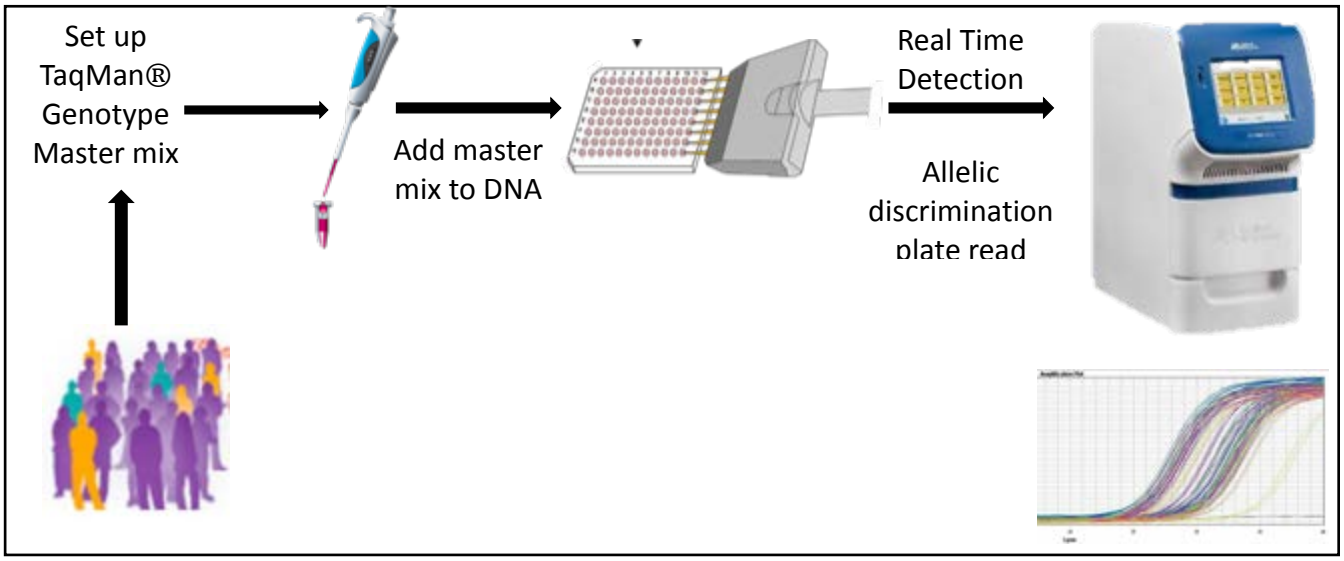

Figure 12 Research Design Schematic SNP Allelic Discrimination (Victor, 2016)

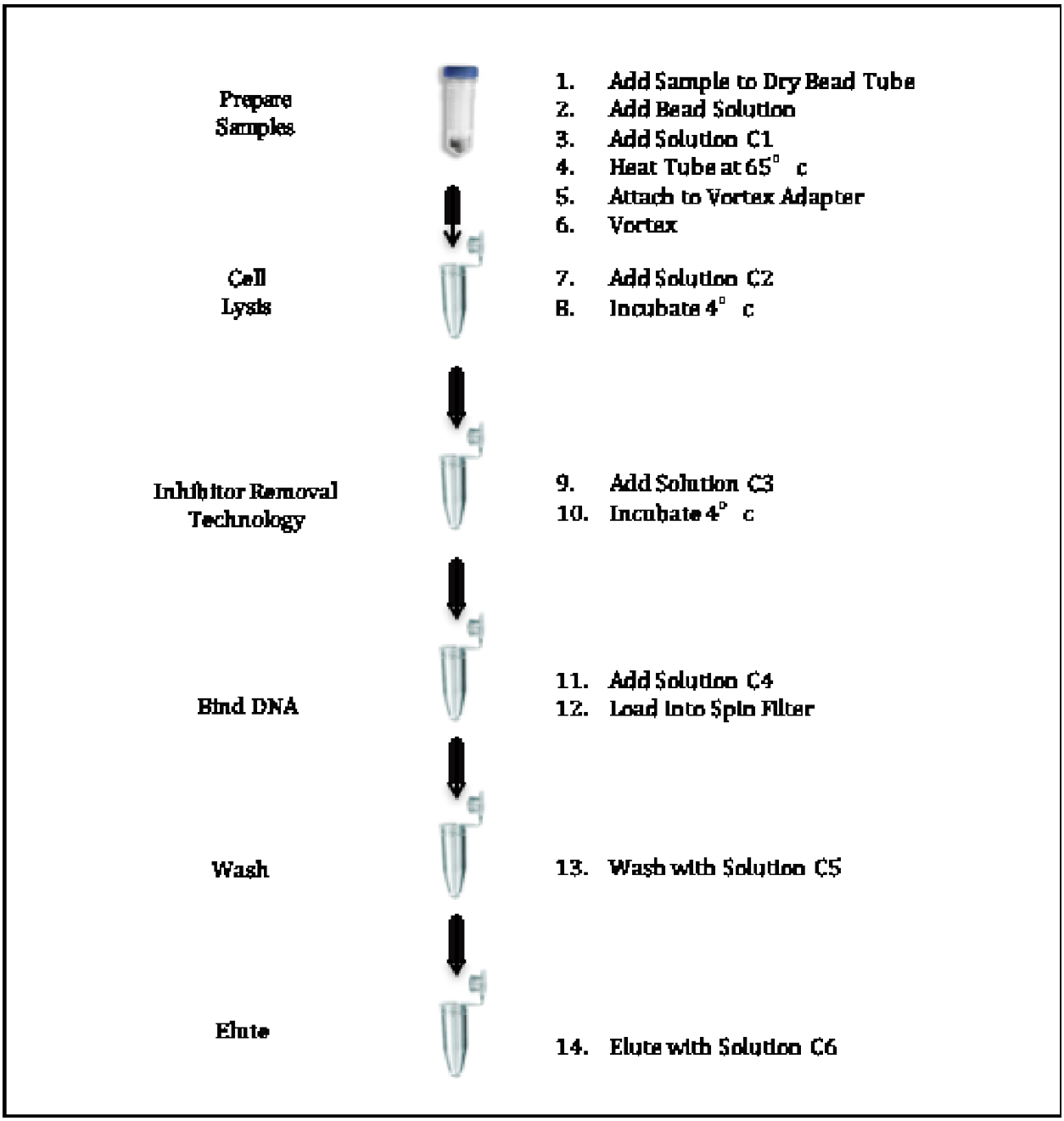

Figure 13 Procedural Schematic of Fecal DNA Isolation (Victor, 2016)

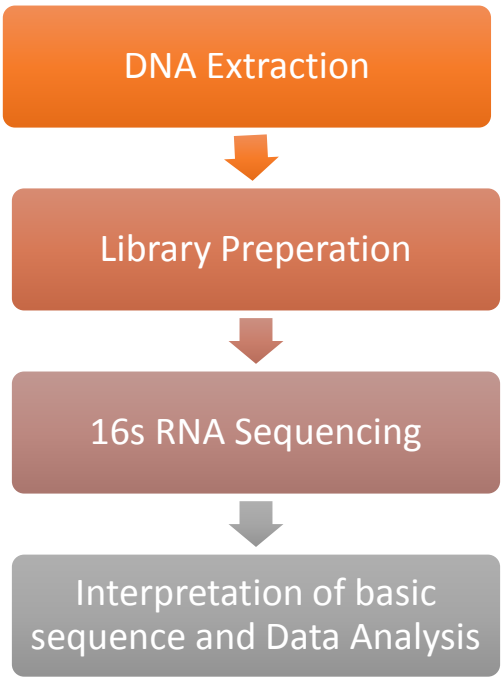

Figure 14 Process to identify microbial products (Victor, 2016)

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